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The nuclear F-actin interactome of *Xenopus* oocytes reveals an actin-bundling kinesin that is essential for meiotic cytokinesis

Matthias Samwer, Heinz-Jürgen Dehne, Felix Spira, Martin Kollmar, Daniel W. Gerlich, Henning Urlaub, Dirk Görlich

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

12 April 2013

Thank you for submitting your manuscript on nuclear F-actin interactors and the identification of NabKin for consideration by The EMBO Journal. It has now been assessed by three expert referees, all of whom find the work of high interest and in principle suited for publication, pending adequate revision of a number of specific points. We shall therefore be happy to consider a revised version of this study further for publication. As you will see, a number of minor points will need to be clarified or better discussed, while additional experimental data would not be essential at this stage. Two referees question the importance of KIF14 results, and while I agree that these data should be given less emphasis and more cautiously interpreted, I would not follow referee 1's suggestion to completely remove them. Should you have any other questions regarding the referee reports and the revision, please do not hesitate to get back to me.

When preparing your letter of response, please be reminded that our policy to allow only a single round of major revision will necessitate diligent and comprehensive answering, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published here or elsewhere during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider this work for publication. I look forward to your revision.

 REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The initial aim of Samwer and coworkers is to identify the proteins that organize the nuclear F-actin network that is required for the mechanical stabilization of the giant nuclei of *Xenopus* oocytes. For this purpose they establish a novel affinity matrix composed of phalloidin chemically coupled to magnetic beads. They then use this matrix to purify F-actin interactors from manually isolated *Xenopus* nuclei and analyze the samples by mass spectrometry. This resulted in a list of nuclear F-actin regulators very distinct from those that interact with actin filaments in the cytoplasm. Surprisingly, the most prominent hit was a kinesin designated here as NabKin. The authors show that NabKin not only binds microtubules as all kinesins do, but also F-actin. NabKin co-localizes with the bundles of the nuclear F-actin network in oocytes, and upon entry into the meiosis labels principally all microtubule and F-actin structures including the meiotic spindle, the cell cortex, and the contractile ring. It is also prominent on the "cortical cap", the thickening of the cortex above the meiotic spindle (in the manuscript referred to as "roof" that I would propose to change to cortical cap for clarity). The authors also map the region of NabKin that binds F-actin and that is located in its N-terminal extension. Interestingly, this same region is responsible for NabKin's nuclear localization and as shown F-actin binding is also controlled by binding of the nuclear import receptor, importin-beta in a RanGTP-dependent manner. By injection of an inhibitory antibody, the authors also address the cellular functions of NabKin and demonstrate that NabKin is essential for polar body cytokinesis. Furthermore, the authors also identify Kif14 as the somatic variant of the meiosis-specific NabKin, and show that Kif14 in addition to its known microtubule interaction, also binds F-actin. However, Kif14 is distinct from NabKin in that it is cytoplasmic and is therefore not regulated by importins, however similar to NabKin is also essential for cytokinesis of somatic cells.

First, the manuscript describes a novel method for purification of F-actin interactors and demonstrates the utility of this new method. By purifying F-actin interactors from isolated *Xenopus* nuclei the authors for the first time reveal an inventory of nuclear F-actin regulators. Second, they identify a new protein, NabKin that turns out to be a novel and essential regulator of polar body cytokinesis. In addition NabKin is a very interesting and in many aspects novel and unique protein to link the actin and microtubule cytoskeleton and is regulated by the Ran GTPase system. All these results are presented in a well-written manuscript and the experimental design as well as the presentation of the results on the figures is extremely clear and convincing. In fact, this many results would even suffice for 2-3 separate papers, and thus I can only recommend this manuscript for publication in The EMBO Journal.

Indeed, my only criticism would be that the large number of experiments makes the manuscript complicated. For example, I wonder whether the data on Kif14 in somatic cells is absolutely necessary to show. An alternative would be to focus more strictly on the functions of NabKin in oocytes that may make the manuscript easier to follow. In this regard, I also wonder whether the authors have seen any effects of the NabKin antibody injection on the nuclear F-actin network. As the original purpose of the study was to identify regulators of this nuclear F-actin network, it would be interesting to know whether NabKin inactivation causes any mechanical defects similar to those caused by exportin-6 expression in oocytes in a previous study of the authors.

Referee #2 (Remarks to the Author):

The manuscript by Samwer et.al. examines the content of actin-binding proteins within nuclei of *Xenopus* oocytes. Oocytes were manually dissected into nuclear and cytoplasmic fractions. These fractions were then subjected to pull down experiments using a novel approach with phalloidin-coated beads. The precipitated actin-associated proteins were then subjected to mass spectrometry analysis. This analysis revealed an unexpected binding interaction between actin and an actin bundling kinesin, here termed NabKin, with homology to KIF14. The authors proceed to

characterize this protein in terms of domain analysis, bundling activity using in vitro actin assays, localization within fixed oocyte nuclei, and localization within a tissue culture system. These results elucidate a role for Nabkin in linking actin and microtubules during cytokinesis; disrupting Nabkin/actin interactions led to cytokinesis failure. In addition to revealing the identity and function of a novel nuclear actin binding protein, this study shows that actin within oocyte nuclei exhibits a distinct and unexpected network of interactions, underscoring the unusual nature of this oocyte nuclear actin. This work represents a comprehensive analysis utilizing a variety of complementary cell and molecular biological assays, and is an important contribution to the field.

One question that I have concerns the longstanding controversy over the nature of actin within nuclei in general, and oocyte nuclei in particular. As pointed out in the introduction, several authors have questioned the nature of actin inside oocyte nuclei, suggesting it may not be in a truly filamentous form under native conditions. Part of these concerns have been based on the potential for perturbation of nuclear actin upon dissection into aqueous buffer. It was not clear to me from the text how the nucleus is dissected; this should be mentioned more explicitly, and any controls that have been done (?) that argue against such effects.

Another related point which was not clear to me from the text was why the binding was performed "at high ATP-level". Isn't it possible that the ATP levels could be altering the polymerization state of actin?

Referee #3 (Remarks to the Author):

Samwer et al. develop a novel strategy for the analysis of actin binding proteins using immobilised phalloidin as bait. They use this strategy to purify and enrich actin binding proteins from *Xenopus* oocyte nuclei and cytoplasm. This strategy is remarkable and represents a major technical advancement in the field. The authors show a clearly different pattern of actin binding proteins from *Xenopus* cytoplasm and nucleoplasm. They finally identify NabKin, an unusual kinesin of the Kif3 family, as a novel, nuclear specific F-actin interaction partner. After initial identification from the pool of nuclear actin binding proteins, the authors demonstrate actin bundling activity of the N-terminus of NabKin in vitro. They further perform a thorough description of NabKin localisation through meiosis I and II in *Xenopus* oocytes. To address the functional significance of their observation, the authors inject Fab fragments into maturing *Xenopus* oocytes, which efficiently block polar body extrusion but leaves large or tandem spindles with non-segregated chromosomes.

The technical quality of the paper is outstanding and all the presented results are perfectly documented. Although a detailed understanding into the mode of action of NabKin in meiotic spindle function and polar body extrusion is missing, the identification and initial characterisation of this unusual motor protein certainly deserves, to my opinion, publication in EMBO J.

Before publication, however, the following issues should be addressed/discussed:

Major issues:

The significance of NabKin being both a microtubule motor and an actin bundling protein at the same time was not clearly worked out. Most NabKin associates with the cortex, is MT binding needed to localise NabKin to the spindle and if so, what is its function here? The Fab fragment injection experiment leaves spindles with unsegregated bivalents (?). Polar body extrusion, i.e. cytokinesis, is grossly affected although the spindle stays associated with the cortex. Does this mean that chromosomes segregated in meiosis I but were re-collected again in the metaphase II spindle? Along those lines: As stated, NabKin is expressed in all oocyte stages as well as down to the point of midblastula transition, i.e. long after polar body extrusion. This implies a function of NabKin in blastomere cleavage divisions, what about a possible nuclear localisation in blastomere nuclei? This should be at least discussed. I think further experiments in cell free extracts are beyond the scope of this study but may help in the future to clarify a potential function of NabKin in spindle formation and chromosome segregation.

I am not sure in how far the KIF14 experiments can be fully drafted to understanding the molecular

details of NabKin action; as the authors show in Fig. 3a, the importin/Ran responsive module is missing in Kif14 (both human and *Xenopus*). Maybe I overlooked it, but is there any information on other vertebrate orthologs of NabKin? How do they relate in sequence to the *Xenopus* ortholog? The discussion on this issue is not clear enough.

Minor issues:

Figure 2c: "binding/bundling"

Figure 2e: Please indicate from where in the larger pictures the insets were taken.

To my opinion, Fig. S2 does not add to the story and may be left out.

1st Revision - authors' response

18 April 2013

Reply to points raised by the Reviewers

For the sake of clarity, we repeat the raised points in blue in front of each of our answers.

Referee #1

The initial aim of Samwer and coworkers is to identify the proteins that organize the nuclear F-actin network that is required for the mechanical stabilization of the giant nuclei of *Xenopus* oocytes. For this purpose they establish a novel affinity matrix composed of phalloidin chemically coupled to magnetic beads. They then use this matrix to purify F-actin interactors from manually isolated *Xenopus* nuclei and analyze the samples by mass spectrometry. This resulted in a list of nuclear F-actin regulators very distinct from those that interact with actin filaments in the cytoplasm. Surprisingly, the most prominent hit was a kinesin designated here as NabKin. The authors show that NabKin not only binds microtubules as all kinesins do, but also F-actin. NabKin co-localizes with the bundles of the nuclear F-actin network in oocytes, and upon entry into the meiosis labels principally all microtubule and F-actin structures including the meiotic spindle, the cell cortex, and the contractile ring. It is also prominent on the "cortical cap", the thickening of the cortex above the meiotic spindle (in the manuscript referred to as "roof" that I would propose to change to cortical cap for clarity). The authors also map the region of NabKin that binds F-actin and that is located in its N-terminal extension. Interestingly, this same region is responsible for NabKin's nuclear localization and as shown F-actin binding is also controlled by binding of the nuclear import receptor, importin-beta in a RanGTP-dependent manner. By injection of an inhibitory antibody, the authors also address the cellular functions of NabKin and demonstrate that NabKin is essential for polar body cytokinesis. Furthermore, the authors also identify Kif14 as the somatic variant of the meiosis-specific NabKin, and show that Kif14 in addition to its known microtubule interaction, also binds F-actin. However, Kif14 is distinct from NabKin in that it is cytoplasmic and is therefore not regulated by importins, however similar to NabKin is also essential for cytokinesis of somatic cells.

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Thank you very much!

Indeed, my only criticism would be that the large number of experiments makes the manuscript complicated. For example, I wonder whether the data on Kif14 in somatic cells is absolutely necessary to show. An alternative would be to focus more strictly on the functions of NabKin in oocytes that may make the manuscript easier to follow.

The KIF14 dataset is not essential. However, it extends our findings on NabKin's role in meiosis to somatic cell cycles and from amphibians to mammals. In addition, it helps putting the NabKin data into the context of the existing literature and unifies critical aspects of KIF14 and NabKin function. We therefore prefer to keep this dataset. However, we optimised the manuscript again for readability and removed a number of potentially distracting details on KIF14 function from the Discussion.

In this regard, I also wonder whether the authors have seen any effects of the NabKin antibody injection on the nuclear F-actin network. As the original purpose of the study was to identify

regulators of this nuclear F-actin network, it would be interesting to know whether NabKin inactivation causes any mechanical defects similar to those caused by exportin-6 expression in oocytes in a previous study of the authors.

We agree that this is a very interesting question. However, we have not yet done the experiment, mainly for the reason that the oocyte nucleus contains several F-actin bundling and crosslinking proteins (NabKin, Supravillin, and Filamin A), which predicts that inhibition of NabKin alone will be insufficient for disrupting the entire scaffold.

Referee #2:

The manuscript by Samwer et.al. examines the content of actin-binding proteins within nuclei of *Xenopus* oocytes. Oocytes were manually dissected into nuclear and cytoplasmic fractions. These fractions were then subjected to pull down experiments using a novel approach with phalloidin-coated beads. The precipitated actin-associated proteins were then subjected to mass spectrometry analysis. This analysis revealed an unexpected binding interaction between actin and an actin bundling kinesin, here termed NabKin, with homology to KIF14. The authors proceed to characterize this protein in terms of domain analysis, bundling activity using in vitro actin assays, localization within fixed oocyte nuclei, and localization within a tissue culture system. These results elucidate a role for Nabkin in linking actin and microtubules during cytokinesis; disrupting Nabkin/actin interactions led to cytokinesis failure. In addition to revealing the identity and function of a novel nuclear actin binding protein, this study shows that actin within oocyte nuclei exhibits a distinct and unexpected network of interactions, underscoring the unusual nature of this oocyte nuclear actin. This work represents a comprehensive analysis utilizing a variety of complementary cell and molecular biological assays, and is an important contribution to the field.

Thank you for this very positive evaluation!

One question that I have concerns the longstanding controversy over the nature of actin within nuclei in general, and oocyte nuclei in particular. As pointed out in the introduction, several authors have questioned the nature of actin inside oocyte nuclei, suggesting it may not be in a truly filamentous form under native conditions. Part of these concerns have been based on the potential for perturbation of nuclear actin upon dissection into aqueous buffer. It was not clear to me from the text how the nucleus is dissected; this should be mentioned more explicitly, and any controls that have been done (?) that argue against such effects.

We did not mean to suggest that we isolated the undisturbed intranuclear actin structure from oocyte nuclei (which probably would be an impossible undertaking). Instead, we isolated F-actin binders by a chromatographic procedure that requires soluble proteins as a starting material. For that, we initially depolymerised the intranuclear actin with Latrunculin A, removed remaining insoluble material by centrifugation and added the phalloidin beads, which then triggered re-polymerisation of actin and acted as the affinity matrix. These details were so far only listed in Materials and methods. We now include a more detailed description of the procedure also into the main text (Page 6). Thank you for pointing to this problem.

Concerning the discussion about presence of F-actin filaments in alive oocyte nuclei and a potentially perturbing role of aqueous isolation buffers: We now include the additional Supplementary Fig. 9. It shows the Lifeact-GFP-stained intranuclear F-actin network of an unfixed oocyte. The imaged nucleus had been isolated in mineral oil and had therefore no contact to any exogenous buffer. This further adds to the line of arguments for the presence of filamentous actin in the nucleus of *Xenopus* oocytes that is already presented in the paper.

Another related point which was not clear to me from the text was why the binding was performed "at high ATP-level". Isn't it possible that the ATP levels could be altering the polymerization state of actin?

We actually meant something different, namely a high ATP: ADP ratio (of probably $\approx 100: 1$), maintained by a creatine phosphate-based energy-regenerating system. The buffer contained 1 mM ATP. Both parameters approximate the conditions found in live cells. We now clarified the text accordingly. Thank you for spotting the unclear wording. And yes, ATP-actin is the preferred substrate of polymerisation.

Referee #3:

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Major issues:

The significance of NabKin being both a microtubule motor and an actin bundling protein at the same time was not clearly worked out. Most NabKin associates with the cortex, is MT binding needed to localise NabKin to the spindle and if so, what is its function here?

NabKin contains a conserved kinesin motor domain, and the simplest explanation is that this domain directly mediates the observed binding of NabKin to individual microtubules, the transient microtubule array and the meiotic spindle. However, this assumption is very difficult to test in the setting of a *Xenopus* oocyte. Likewise, we cannot tell if NabKin has a specific function at the meiotic spindle.

The Fab fragment injection experiment leaves spindles with unsegregated bivalents (?). Polar body extrusion, i.e. cytokinesis, is grossly affected although the spindle stays associated with the cortex. Does this mean that chromosomes segregated in meiosis I but were re-collected again in the metaphase II spindle?

We fully agree with the latter interpretation of the NabKin inhibition experiment. This interpretation is backed by the observation of twinned spindles in anti-NabKin treated oocytes, which represents a phenotype that can only be explained if bivalents had been segregated in the first place. As *Xenopus* oocyte probably do not have an active spindle checkpoint during meiosis I (Peter *et al*, 2001; Shao *et*

al, 2013), this is also in line with the expected behaviour of the oocyte.

Along those lines: As stated, NabKin is expressed in all oocyte stages as well as down to the point of midblastula transition, i.e. long after polar body extrusion. This implies a function of NabKin in blastomere cleavage divisions, what about a possible nuclear localisation in blastomere nuclei? This should be at least discussed.

This addresses an interesting point. Nevertheless, we do not yet know if NabKin or KIF14 play a role during blastomere cleavage divisions. There is certainly a transition between the two proteins at some point, but it is unclear when this happens. The Western blot of Fig. 1f only predicts that this transition should occur not later than midblastula transition (MBT), however, it is possible that NabKin becomes dispensable already right after meiosis.

We find it difficult to believe that NabKin is continuously expressed between fertilisation and MBT. We would rather assume that the already existing NabKin pool is not degraded before MBT. We do not know how this persisting pool is distributed between polar bodies and the egg, but the suggested experiment of staining blastomere stages for NabKin could provide an answer. However, we have not yet done the experiment. Likewise, we do not yet know at which stage the expression of KIF14 is initiated.

As reviewer #1 (rightly) felt that the manuscript is already pretty complex, and we would prefer not to add another chapter to the Discussion. However, as this conversation will be part of the published manuscript, this should not be a serious problem.

I think further experiments in cell free extracts are beyond the scope of this study but may help in the future to clarify a potential function of NabKin in spindle formation and chromosome segregation.

Indeed, extracts from non-activated eggs will be a great tool to address a potential function of NabKin at the second meiotic spindle.

I am not sure in how far the KIF14 experiments can be fully drafted to understanding the molecular details of NabKin action; as the authors show in Fig. 3a, the importin/Ran responsive module is missing in Kif14 (both human and *Xenopus*).

We already carefully considered similarities and differences between NabKin and KIF14. The two share a similar modular structure, an actin-binding module, localisation to the contractile actomyosin ring and an essential role during cytokinesis. Indeed, NabKin acquired an additional layer of regulation, namely by importin beta, but we think that this is adequately discussed in the manuscript. However, we now removed the comparison between NabKin and KIF14 in respect to regulation by citron kinase and PRC1, as these are only indirectly covered by our experimental data.

Maybe I overlooked it, but is there any information on other vertebrate orthologs of NabKin? How do they relate in sequence to the *Xenopus* ortholog? The discussion on this issue is not clear enough.

We agree and added now the following paragraph to the Discussion: "The functional specialisation between a somatic KIF14 and the female-meiotic NabKin appears specific to the amphibian lineage. This predicts that KIF14 of other metazoans functions not only during mitotic cell division, but may also play a role during meiotic polar body extrusion. The consideration begs interesting questions, namely if, and how KIF14 from non-amphibian species can switch between a meiotic and a somatic mode of regulation."

Minor issues:

Figure 2c: "binding/bundling"

Thanks, typo has been corrected.

Figure 2e: Please indicate from where in the larger pictures the insets were taken.

We now indicate this in Fig. 2e

To my opinion, Fig. S2 does not add to the story and may be left out.

Figure S2 contains a quite informative characterisation of our new phalloidin affinity matrix. It demonstrates that also myosins can be co-purified with actin on these beads and that the interaction shows the expected ATP-sensitivity. We feel that the figure should stay in the manuscript, but we have simplified it significantly.

References:

Peter M, Castro A, Lorca T, Le Peuch C, Magnaghi-Jaulin L, Dorée M & Labbé JC (2001) The APC is dispensable for first meiotic anaphase in *Xenopus* oocytes. *Nat Cell Biol* **3**: 83–87

Shao H, Li R, Ma C, Chen E & Liu XJ (2013) *Xenopus* oocyte meiosis lacks spindle assembly checkpoint control. *J Cell Biol* **201**: 191–200

Acceptance letter

23 April 2013

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that there are no further objections towards publication in *The EMBO Journal*.